

Blastomere biopsy for PGD delays embryo compaction and blastulation: a time-lapse microscopic analysis

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Abstract

Purpose The purpose of the study was to explore the effect of blastomere biopsy for preimplantation genetic diagnosis (PGD) on the embryos' dynamics, further cleavage, development, and implantation.

Methods The study group included 366 embryos from all PGD treatments (September 2012 to June 2014) cultured in the EmbryoScope™ time-lapse monitoring system. The control group included all intracytoplasmic sperm injection (ICSI) embryos cultured in EmbryoScope™ until day 5 during the same time period (385 embryos). Time points of key embryonic events were analyzed with an EmbryoViewer™.

Results Most (88 %) of the embryos were biopsied at ≥ 8 cells. These results summarize the further dynamic development of the largest cohort of biopsied embryos and demonstrate that blastomere biopsy of cleavage-stage embryos significantly delayed compaction and blastulation compared to the control

non-biopsied embryos. This delay in preimplantation developmental events also affected postimplantation development as observed when the dynamics of non-implanted embryos (known implantation data (KID) negative) were compared to those of implanted embryos (KID positive).

Conclusion Analysis of morphokinetic parameters enabled us to explore how blastomere biopsy interferes with the dynamic sequence of developmental events. Our results show that biopsy delays the compaction and the blastulation of the embryos, leading to a decrease in implantation.

Keywords Embryo development · Blastomere biopsy · Morphokinetics · Time-lapse microscopy · PGD

Introduction

Preimplantation genetic diagnosis (PGD) is offered to couples that carry mutations for severe genetic abnormalities in order to diagnose and select unaffected embryos for transfer into the uterus [1]. Preimplantation genetic screening (PGS) is offered mainly to couples with repeated implantation failure or advanced maternal age in order to select euploid embryos for transfer and thereby increase the chance for a normal pregnancy and birth [2]. Both PGD and PGS require access to the DNA of the preimplanted embryos, which is provided by either polar body biopsy, blastomere biopsy of cleavage-stage embryos, or trophoctoderm biopsy of blastocysts. PGS is mainly performed on trophoctodermal cells biopsied from blastocysts to allow a better representation of the embryo ploidy [3–7]. In contrast, PGD for severe monogenic disorders is usually performed on blastomeres biopsied from day 3 embryos. According to ESHRE PGD Consortium data collection XII (2014), cleavage-stage biopsies accounted for almost 90 % of all biopsy procedures performed in Europe between

Capsule Morphokinetic analysis demonstrated that blastomere biopsy interferes with the dynamic sequence of developmental events, delaying compaction and blastulation of the embryos and leading to a decrease in implantation rate.

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2009 and 2010, while TE biopsy accounted for less than 1 % [6]. The same data referring to years 2012–2013 revealed a shift toward trophectoderm biopsies for PGD as cleavage-stage biopsies decreased to approximately 75 % of all embryo biopsy cycles [reviewed by [8]]. Despite the notable shift, according to the latest ESHRE PGD Consortium data collection XIII published on 2015, cleavage-stage biopsy is currently still the preferential stage/method for biopsy [9].

A recent study by Shapiro et al. demonstrated that embryo freezing does not necessarily decrease implantation potential [10], supporting the shift toward day 5 blastocyst biopsy rather than cleavage-stage biopsy. Mastenbroek et al.'s [11] prospective randomized study also questioned the effectiveness of cleavage-stage biopsy and showed that blastomere biopsy may be considered a somewhat harmful procedure. Polar body biopsy has been proposed as an alternative to embryo biopsy, especially for aneuploidy testing. However, no sufficiently powered study has thus far clarified the impact of this procedure on embryo reproductive competence, and it is not a common practice (approximately 2 % of embryo biopsies) [8].

Blastomere biopsy for PGD is an invasive procedure that involves disruption of cell adhesion and breaching of the zona pellucida followed by aspiration of one or two blastomeres from cleavage-stage embryos. The biopsied blastomeres are then taken for single-cell DNA analysis of a disease-causing mutation together with several flanking polymorphic markers that assist in identifying the allele (maternal or paternal) that had been inherited by the embryo. Embryo transfer following PGD requires the selection of the embryo/s with the highest implantation potential from among the genetically diagnosed healthy embryos. In most centers, this selection is based upon evaluations at only two discrete time points, i.e., day 4 and/or 5 ("static morphological evaluation"). The standard morphologic criteria include the addition of cells following biopsy (the occurrence of cleavage following biopsy), the presence or absence of compaction, the formation of blastocysts, and the grading of the embryo [12]. Studies using static evaluation of mouse embryos following blastomere biopsy showed that the procedure resulted in an earlier compaction [13] and yielded evidence of developmental delay in both mouse and human [14, 15] embryos. Scott et al. reported a significant reduction in implantation potential following cleavage-stage biopsy [4]. Their paired RCT study also used the standard morphologic criteria for the selection of the embryos for transfer. During the last 5 years, image capturing with time-lapse devices was introduced into in vitro fertilization (IVF) labs, offering a non-invasive method to monitor and analyze the full course of embryo development in real time without interfering with the optimal culture conditions [16, 17]. Morphokinetic studies have already shown that embryos with high implantation potential cleave in specific patterns and similar timings [18–23]. Those reports demonstrated that morphokinetic evaluation by time-lapse imaging can provide a more comprehensive and detailed analysis of the developmental events than that of

static evaluation and thus may assist in selecting top quality embryos for transfer. Other studies, however, have questioned the benefit of the time-lapse system over the traditional incubators and assessment grading systems [24–29].

The effect of blastomere biopsy on subsequent embryonic dynamics by using time-lapse microscopy was analyzed in only one pioneer publication by Kirkegaard et al. [30]. The results of that study demonstrated that blastomere removal prolongs the duration of the exact cell stage during which the biopsy was performed, causing biopsied embryos to reach subsequent embryonic stages at significantly later time points. As a result, the time of compaction and blastulation was also delayed in the biopsied embryos compared to non-biopsied control embryos. However, only 56 biopsied embryos were compared with 53 control embryos, thus warranting further investigation to validate the findings. In order to confirm and support those results, we explored the effects of blastomere biopsy on a much larger group of embryos. Furthermore, we aimed to understand the effect of the delay caused by the biopsy on the chances for implantation by analyzing implanted vs. non-implanted embryos. To the best of our knowledge, there is no publication on time-lapse parameters as predictors of implantation following embryo biopsy.

The aim of this study was, therefore, to further explore the effect of blastomere biopsy for preimplantation genetic diagnosis (PGD) on the embryos' dynamics and likelihood of implantation.

Materials and methods

Study population and design

All of the embryos in this study were fertilized by intracytoplasmic sperm injection (ICSI) and cultured in the EmbryoScope™, an incubator with a built-in microscope and camera.

The study group consisted of all embryos from women who underwent PGD treatment at our unit from September 2012 to June 2014 (317 cycles, 3865 embryos). Inclusion criteria were all embryos from those PGD cycles that underwent blastomere biopsy on day 3 (at 67–73 h following ICSI), that were at the six-cell to nine-cell stage at the time of biopsy, and that were cultured in the EmbryoScope™ until embryo transfer. Exclusion criteria were low-grade embryos that were not suitable for blastomere biopsy at day 3 (i.e., <6 cells or with >20 % fragmentation). The study group for all analyses, therefore, includes 366 biopsied embryos from 127 cycles (Table 1). The indications for blastomere biopsy for PGD in the study group were mostly monogenic diseases (66 % of the cycles), chromosomal translocations (32 % of the cycles), and sex selection for X-linked diseases with non-identified mutation (2 % of the cycles).

The control group was comprised of all embryos from cycles performed during the same time period that underwent

Table 1 Baseline and cycle characteristics in PGD and control groups

	Control (no biopsy)		PGD (biopsy)		<i>P</i> value (<i>t</i> test)
Number of treatment cycles and embryos	143 cycles, 385 embryos		127 cycles, 366 embryos		
Maternal age	33.4 ± 0.25		33.1 ± 0.3		NS
Maternal BMI	24.2 ± 6.0		24.6 ± 3.4		NS
Number of retrieved oocytes/cycle	17.4 ± 8.9		15.4 ± 8.2		<i>P</i> < 0.05
Number of mature M2 oocytes	14.0 ± 7.8		13.1 ± 6.7		NS
Number of 2PN	11.3 ± 6.7		10.5 ± 5.6		NS
Number of IVF procedures/patient	2.2 ± 2.6		3 ± 2.9		<i>P</i> < 0.05
Indications for treatment	Male factor	309 (80 %)	Monogenic disease	240 (66 %)	
	Unexplained	49 (13 %)	Chromosomal abnormality	117 (32 %)	
	Tubal factor	14 (4 %)	Sex selection	9 (2 %)	
	Age	4 (1 %)			
	Endometriosis	2 (0.5 %)			
	Missing data	7 (1.5 %)			

Data is presented as mean ± SD

ICSI without a biopsy (405 cycles, 3194 embryos), was cultured in the EmbryoScope™ until day 5, and had a recorded blastulation (143 cycles, 385 embryos). The indications for ICSI in the control group were mostly male factor (80 %), unexplained infertility (13 %), and previous cycles of failed fertilization (7 %).

Maternal age and BMI were similar in both groups. The number of mature oocytes and the resulting embryos (2PN) was also similar (*P* > 0.05), although more oocytes were retrieved in the control group. Taken together, these data demonstrate that the two groups are comparable with regard to embryo development. Baseline and cycle characteristics are presented in Table 1.

The study was approved by the ethics committee of Tel Aviv Medical Center, and institutional review board approval for retrieving IVF data was obtained (0748/15).

Ovarian stimulation, fertilization, and embryo culture

Controlled ovarian stimulation was carried out by either the long gonadotropin releasing hormone (GnRH) agonist, short GnRH agonist, or GnRH antagonist protocols. The long protocol began with the administration of subcutaneous injections of 0.1 mg/day of the GnRH- α triptorelin (Decapeptyl; Ferring, Kiel, Germany) for at least 14 days, followed by concomitant recombinant follicle-stimulating hormone (rFSH; Gonal F [Serono, Geneva, Switzerland] or Puregon [Organon, Oss, The Netherlands]), human menopausal gonadotrophin (hMG; Menogon, Ferring, Kiel, Germany), or highly purified human menopausal gonadotropin (Menopur, Ferring Pharmaceuticals, Geneva, Switzerland). The short protocol began with the administration of the GnRH- α from the first day of the cycle followed by concomitant daily rFSH and

GnRH- α from day 3 of the cycle. In the antagonist protocol, stimulation started with the administration of gonadotropins from days 2 to 3 of the cycle. GnRH antagonist (0.25 mg of cetrorelix acetate, Cetrotide®, Serono or Ganirelix, Orgalutran®, Merck and Co., Inc.) was started when the leading follicle was ≥ 12 mm or the estradiol level was >450 pg/ml and continued until the day of human chorionic gonadotropin (hCG) administration. Choriogonadotropin alfa 250 mcg (Ovitrelle; Serono, Geneva, Switzerland) was administered when at least three follicles achieved an 18-mm diameter. Ovum pickup was performed 36 h later.

The cumulus-oocyte complexes were isolated into modified human tubal fluid (mHTF) (Irvine Scientific). Sperm samples were treated with mHTF medium (Irvine Scientific), and insemination was performed by ICSI at 2–4 h following oocyte retrieval. Oocytes were denuded of cumulus cells by hyaluronidase and a fine pipette. ICSI was performed by means of a Nikon inverted microscope (Diaphot 300; Nikon) with Narishige micromanipulators. Each embryo was incubated in a separate droplet of human embryo culture medium covered with paraffin oil in an EmbryoSlide® culture dish (FertiLife) to allow individual assessment and documentation. Incubation in the EmbryoScope™ incubator lasted from day 1 following ICSI and continued up to day 5 of development.

Blastomere biopsy for PGD

Blastomere biopsy for PGD was performed 67–73 h after ICSI. Prior to biopsy, the embryos were incubated for 2–5 min in Ca²⁺/Mg²⁺-free bicarbonate-buffered medium (G-PGDw Vitrolife, Sweden) in order to loosen cell-to-cell adhesions. A laser was used to create an ~30- μ m opening in the

zona pellucida (Zilos™, Hamilton Thorne Research, wavelength 1480 nm), and one or two blastomeres were gently biopsied by an aspiration pipette through the hole. After biopsy, the embryos were immediately returned to the EmbryoScope™ until transfer.

The policy for blastomere biopsy in our unit is that one blastomere is biopsied in all genetic indications except for translocations or de novo monogenic mutations for which we prefer two blastomeres for the genetic analysis whenever available (i.e., good quality embryo with >7 cells). However, when only lower quality embryos are available and the embryo is <7 cells, only one blastomere is biopsied.

Time-lapse monitoring of embryo morphokinetics

All embryos were incubated in the integrated EmbryoScope™ time-lapse monitoring system (EmbryoScope™; Unisense FertiTech A/S, Aarhus, Denmark, Vitrolyfe) from the time of ICSI until embryo transfer on days 4–5. The EmbryoScope™ offers the possibility of continuous monitoring of embryo development without disturbing the culture conditions. Embryo scoring and selection with time-lapse monitoring were performed by analysis of time-lapse images of each embryo on an external computer with software developed specifically for image analysis (EmbryoViewer workstation; Unisense FertiTech A/S). Embryo morphology and developmental events were recorded to demonstrate the precise timing of the observed cell divisions in correlation to the timing of ICSI: time of cleavage to a two-blastomere (t_2), three-blastomere (t_3), four-blastomere (t_4), and so forth until reaching a nine-blastomere (t_9) embryo. Timing of first signs of compaction that indicated the first time point at which cell boundaries could no longer be clearly seen was also recorded (t_M). The first signs of the beginning of blastulation (t_{SB}) were recorded as soon as a star-shaped space was demonstrated in the compacted embryo, and the first sign of viability following biopsy was documented when the first cleavage after biopsy was observed (t_{FCAB}). Embryos that were transferred on day 5 were annotated for t_{FCAB} , t_M , and t_{SB} , whereas embryos that were transferred on day 4 were annotated solely for t_{FCAB} and t_M . All the assessments and annotations of the embryos were performed by senior embryologists, ensuring a very low inter-observer variation.

Statistical methods

Maternal age was compared by using t test. Since the distribution of the embryonal measurements (timing of events) was not normal in any of the developmental parameters, the Wilcoxon sum ranked test was used for comparing between the study and control groups and chi-squared test to compare the proportions between the groups. Data are presented as median and interquartile range (IQR; Q1, Q3). A P value of

0.05 was considered statistically significant. Statistical analysis was performed by SAS for Windows version 9.4.

Results

A total of 751 embryos were studied, including 366 embryos that were biopsied at day 3 for the purpose of PGD (study group) and 385 ICSI embryos that did not undergo biopsy (control group). Biopsy was always performed at 67–73 h after ICSI, and most of the embryos (88 %) were biopsied at ≥ 8 -cell stage (Fig. 1). The study and control groups were similar in maternal age (33.1 ± 0.3 vs. 33.4 ± 0.25 years, respectively; t test; $P > 0.05$).

In order to analyze the effect of blastomere biopsy on the timing of embryonic events, we first confirmed that the study and control groups had a similar preinterference developmental rate. There were no differences in the prebiopsy parameters between the study and control groups (Supplemental Table 1). The effect of blastomere biopsy on the subsequent embryonic development was first analyzed on all biopsied embryos by comparing two postbiopsy embryonic stages: the first signs of compaction (t_M) and the time of blastulation (t_{SB} , defined in **Materials and methods** section). When analyzing the median timing of the postbiopsy events, the results showed that a blastomere biopsy significantly delays the compaction of biopsied embryos compared to the control embryos ($t_M = 83.6$ (IQR 77.8 to 89.7) h vs. 79.5 (IQR 72.9 to 86.9) h, respectively; $P < 0.001$). A recovery of the developmental rate was observed at the time of blastulation and with no significant difference between the groups [$t_{SB} = 100.6$ (IQR 94.9 to 107.1) h vs. 99.5 (IQR 94.4 to 105.6) h, respectively; $P = 0.16$] (Fig. 2). However, this analysis included embryos that were biopsied at different developmental stages (six-cell to eight-cell stage), which

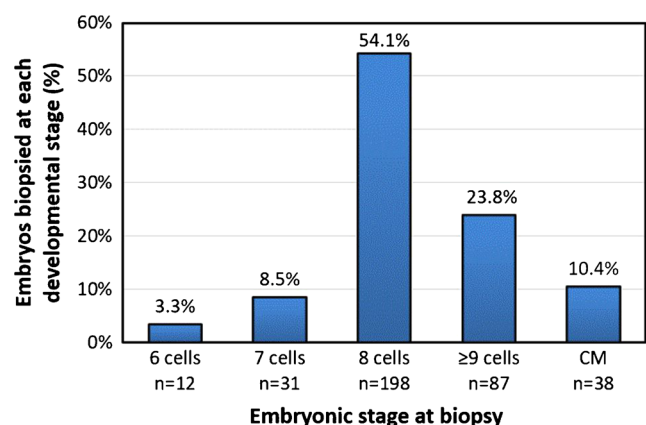


Fig. 1 Embryonic stage at the time of biopsy. Percentage of embryos that were biopsied at each embryonic stage. The blastomere biopsy was performed 67–73 h after insemination. CM compact morula

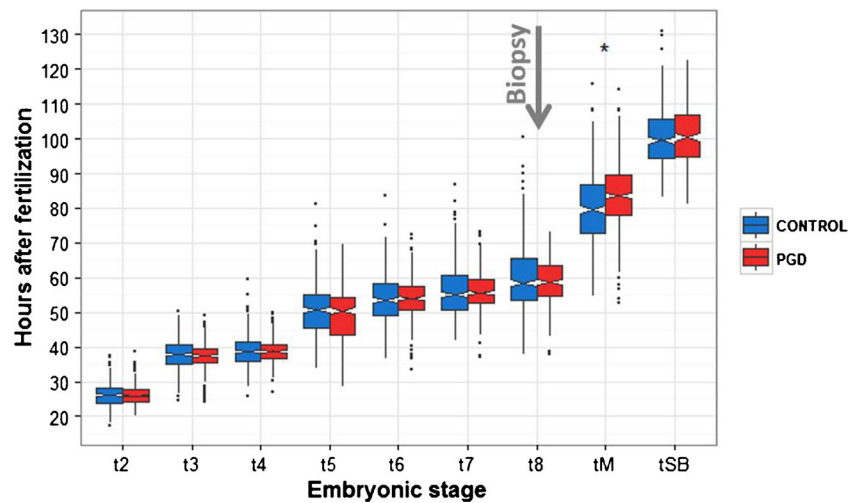


Fig. 2 The effect of blastomere biopsy on the timing of embryonic events. Comparison between the developmental rates of embryos following a blastomere biopsy (preimplantation genetic diagnosis [PGD] group, red line) and the control group (blue line). Blastomere

biopsies were performed on embryos at all cell stages. The notch within the box denotes the median, and the box spans the interquartile range (25–75th percentiles). Start of compaction (t_M); start of blastulation (t_{SB}). * $P < 0.001$, Wilcoxon sum ranked test

may mask the effect of embryo biopsy on the subsequent developmental events.

In order to nullify the differences in the developmental stages of the embryos at the time of biopsy, we analyzed a subgroup of embryos that was biopsied exactly at the eight-cell stage, i.e., the optimal cell stage at the time of biopsy (198 embryos). In order to select a matched control subgroup, we analyzed the mean \pm standard deviation of the biopsy time of the study subgroup and found it to be between 67.8 and 73.1 h after ICSI. The matched control group therefore included non-biopsied embryos that were at the eight-cell stage during the same period of time (67.8–73.1 h after ICSI), meaning that if they were to undergo biopsy, it would have been while they were at the eight-cell stage as well (165 non-biopsied embryos). Comparison of the prebiopsy developmental events demonstrated similarity between these two subgroups in all parameters except for t_5 and the resulting S3 ($S_3 = t_8 - t_5$), showing that the control group was slower to reach t_5 than the study group. It is important to note that the fact that the control embryos were slower, regarding this single parameter, does not hamper our final conclusion but rather strengthen it. It highlights the effect of the biopsy since the study group became significantly slower after the intervention. Given that the vast majority of the prebiopsy parameters were similar between the two groups, it is reasonable to consider both groups as being eligible for such comparison (Supplemental Table 2). The first cleavage after biopsy (t_{FCAB}) in the study subgroup was compared to t_9 in the control subgroup since these two events are expected to be equivalent if a biopsy does not affect the embryonic cell cycle. The results

demonstrated that there was a significant delay already at the time of FCAB/ t_9 [77.5 (IQR 74.3 to 81.6) h vs. 74.1 (IQR 70.8 to 78.4) h in the study and control groups, respectively; $P < 0.001$]. The first sign of compaction of the biopsied embryos was also significantly delayed compared to the non-biopsied embryos [86.1 (IQR 80.8 to 91.2) h vs. 82.6 (IQR 77.5 to 88.7) h in the study and control groups, respectively; $P < 0.001$]. Similarly, the blastulation time was significantly delayed in this more synchronous subgroup of embryos that was all in the eight-cell stage at the time of biopsy [102.1 (IQR 96.5 to 108.4) h vs. 99.9 (IQR 94.9 to 105.4) h in the study and control groups, respectively; $P = 0.038$] (Fig. 3).

In order to evaluate the impact of the delayed preimplantation events observed above on further embryo development postimplantation, we compared the timing of developmental events of PGD embryos with verified implantation (known implantation data, PGD-KID⁺, $n = 22$) to that of PGD embryos that did not implant (PGD-KID⁻, $n = 98$). Our results showed that while the two groups had similar dynamics until the time of biopsy (Supplemental Table 3), the PGD-KID⁻ embryos (non-implanted biopsied embryos) demonstrated a significant delay in both t_M and t_{SB} compared to the PGD-KID⁺ embryos (implanted biopsied embryos), suggesting a correlation between a postbiopsy developmental delay and implantation potential [$t_M = 85.7$ (IQR 79.5 to 89.8) h vs. 77.3 (IQR 73.0 to 85.9) h in the PGD-KID⁻ and PGD-KID⁺ groups, respectively; $P = 0.01$; $t_{SB} = 101.0$ (IQR 95.6 to 109.0) h vs. 93.4 (IQR 92.5 to 97.8) h in the PGD-KID⁻ and PGD-KID⁺ groups, respectively; $P < 0.001$] (Fig. 4).

Analysis of the indications for performing PGD demonstrated that they were similar in the PGD-KID⁺ and the

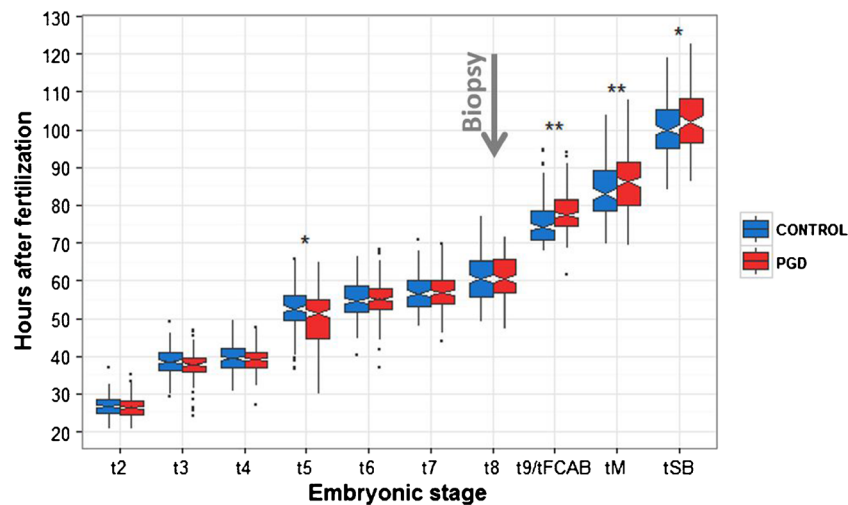


Fig. 3 The effect of blastomere biopsies of eight-cell embryos on the timing of subsequent embryonic events. Comparison between the developmental rates following blastomere biopsies of eight-cell embryos (preimplantation genetic diagnosis [PGD], red line) and a matched control

group (blue line). The notch within the box denotes the median, and the box spans the interquartile range (25–75th percentiles). Start of compaction (t_M); start of blastulation (t_{SB}). * $P < 0.05$, Wilcoxon sum ranked test; ** $P < 0.001$

PGD-KID- groups (monogenic diseases 59 % vs. 70 % respectively; chromosomal abnormalities 41 % vs. 30 % respectively; Chi-square test, $P > 0.05$).

Discussion

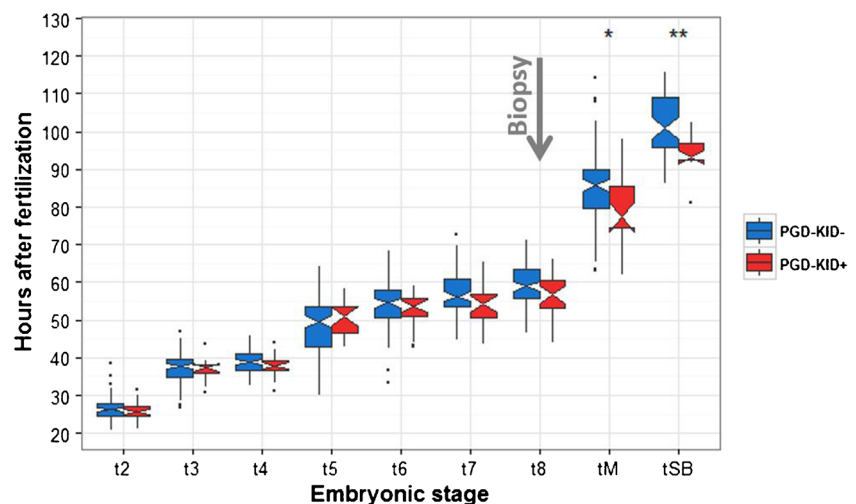
This study comprehensively and systematically analyzes the morphokinetic effect of blastomere biopsy for PGD on the subsequent preimplantation and postimplantation embryonic development by using time-lapse microscopy. Our results demonstrated that blastomere biopsy, including removal of the embryo from the controlled incubation environment within the EmbryoScope™, disruption of cell adhesion, and drilling of the zona pellucida, significantly delayed all postbiopsy developmental events, including the time to the first cleavage

after biopsy, the time to compaction, and the time to blastulation.

A blastomere biopsy had been previously shown to cause developmental delay, as examined by static evaluation, with its well-recognized limitations in analyzing embryo dynamics [14, 15]. However, there is only one publication in which time-lapse microscopy was used to evaluate the effect of blastomere biopsy on subsequent embryonic development although on a much smaller scale [30]. Our sample included a total of 751 embryos, and the findings showed a significant delay not only of the cell stage during which the biopsy was performed but also of all the postbiopsy developmental events. An effect on implantation potential was shown as well.

Time-lapse monitoring and analyses of human IVF embryos have shown that the kinetics of early embryo development is closely related to their potential to develop into blastocysts

Fig. 4 Comparison of postbiopsy developmental events between implanted and non-implanted embryos. Analysis of the implanted embryos (PGD-KID+, 22 embryos, red line) and the non-implanted embryos (PGD-KID-, 98 embryos, blue line). The notch within the box denotes the median, and the box spans the interquartile range (25–75th percentiles). Start of compaction (t_M); start of blastulation (t_{SB}). * $P = 0.01$, Wilcoxon sum ranked test; ** $P < 0.001$



on day 5 [31]. Several studies have shown a correlation between some morphokinetic parameters and the embryo's implantation potential [18–21, 26]. We now show that the postbiopsy morphokinetics of the biopsied embryos that were implanted (PGD-KID⁺) was significantly faster compared to the biopsied PGD-KID⁻ embryos that were not implanted. These results reveal a potential correlation between the developmental delay following blastomere biopsy and low implantation potential. It may demonstrate the embryos' difficulty to overcome the interference of a blastomere biopsy. An interesting explanation may be that embryos at this stage of preimplantation development are relatively fragile since embryonic genome activation and cell differentiation processes have not yet occurred [8]. Thus, downstream developmental processes can be irreparably compromised by removing a cell from the embryo. Such an impact is also reflected in a lower blastocyst rate after cleavage-stage biopsy with respect to undisturbed embryos, as reported in several papers [32–34].

Although the removal of blastomeres from an eight-cell stage embryo is an invasive procedure, mammalian embryos are known to recover from most of such manipulations because their blastomeres can adjust their behavior in response to changing conditions [12]. Our results demonstrated that a blastomere biopsy significantly delayed later embryonic development and that a developmental delay characterizes the non-implanted embryos (PGD-KID⁻) as opposed to the implanted embryos (PGD-KID⁺). These findings suggest that the delay observed is a negative prognostic sign for implantation. However, we noticed that the embryos that succeeded to implant (PGD-KID⁺) were the embryos that had a faster postbiopsy developmental rate, suggesting that those that are able to “catch up” with the normal cleavage rate will probably have a greater chance for implantation.

The limitations of this study arise from the fact that it is retrospective and not a prospective controlled investigation, thereby limiting our ability to isolate the effect of additional exposure of the embryo to the suboptimal conditions of a biopsy procedure. During cleavage-stage biopsies, Ca⁺²/Mg⁺²-free medium is used in order to loosen cell-to-cell adhesion and facilitate selected blastomere removal. Several murine studies showed that Ca⁺² depletion caused remodeling of the cellular cytoskeleton, inevitably affecting compaction [35–38]. Furthermore, artificial breaching of the zona pellucida by means of the laser system to enable blastomere biopsy may increase local temperature for a few milliseconds and potentially impact subsequent development [39], although Taylor et al. had shown that laser pulse does not influence embryo development when the size of the hole is controlled [40–43]. All these interruptions in culture conditions, including the total longer duration outside the incubator, can cause the observed delay in further development. Due to ethical restraints, no prospective study has been conducted on human embryos that could investigate each effect separately.

Therefore, the direct effect of the depletion of embryonic mass on further development is not yet fully understood.

There are several differences between the two patient groups from which the embryos were derived. The most prominent is that patients in the PGD group are basically fertile compared to the infertile control group. However, the fact that embryos from both groups had very similar dynamics of development before biopsy highlights the significant differences that were observed after biopsy, thus suggesting a direct impact of the biopsy itself on subsequent development.

There are scant data on implanted embryos following biopsy of patients with genetic indication since only 50–75 % of these embryos will be eligible for transfer (i.e., healthy). Moreover, single embryo transfer in this group is also not common. Therefore, KID data of PGD embryos are fewer than desired, but they still provide an important clue of how biopsy affects implantation. We acknowledge that a comparison of PGD-KID⁺ with control-KID⁺ could have been of great importance for the implication of blastomere biopsy on implantation; however, it was unavailable since non-biopsied embryos are routinely transferred in our unit on day 3, precluding the availability of KID data for the control embryos (day 5 transfer).

It was recently suggested that a trophectoderm biopsy from a day 5 blastocyst has a less harmful effect on further embryonic development compared to blastomere biopsy of day 3 cleavage-stage embryos [3, 8, 11, 44, 45]. Scott et al. identified blastocyst-stage biopsy as a procedure that does not affect embryo viability and implantation potential as opposed to blastomere biopsy [4]. This may be because a trophectoderm biopsy does not interfere with the inner cell mass (ICM) from which all embryonic tissues will develop and because a smaller portion of the embryo mass is removed. A trophectoderm biopsy is considered to be less invasive than a blastomere biopsy in which progenitors of ICM can be unintentionally removed [12]. It was recently recognized that freezing embryos by using vitrification does not necessarily decrease implantation potential [10]. In addition, the fact that frozen embryo transfers are performed in the absence of ovarian stimulation (lowering the risk for ovarian hyperstimulation syndrome and multiple pregnancies) has led to a shift from day 3 cleavage-stage biopsies to day 5 blastocyst biopsies. Nevertheless, cleavage-stage biopsy is still the most commonly used strategy for PGD, at least in Europe, possibly under the perception that it is less operator dependent and more reproducible [8, 9].

There are many biological processes for which timing is critical, from development to senescence [46]. Timing of cell division is controlled by individual cells during their development in all organisms, and it usually follows a precise schedule that tells them when to divide. Our results suggest that growing embryos for blastomere biopsy in the setting of a time-lapse microscopy system may provide embryologists with morphokinetic parameters that may enable them to better

choose the postbiopsy embryos with the best implantation potential. The present study suggests that a day 3 blastomere biopsy delays morphokinetic development, which probably represents the impeded potential of an embryo to successfully implant. That effect is minimized in embryos that are able to catch up with the normal developmental rate and present a smaller delay. The effect of embryonal mass depletion alone on morphokinetic development as a predictor for implantation can be better obtained from a study in which embryos are randomly selected for biopsy, thus showing a direct effect with no confounders.

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